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Unpairing and gating substrate duplex ends enforces FEN superfamily specificity

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Abstract

Structure-specific 5'-nucleases, exemplified by the flap endonucleases (FENs), are a superfamily of evolutionarily conserved phosphodiesterases that catalyze the precise incision of a diverse range of DNA and RNA substrates. Superfamily members, that include FENs, EXO1, XPG, GEN1 and the XRNs, play key roles in many cellular processes such as DNA replication, DNA repair, recombination, transcription, RNA turnover and RNA interference. In this review we discuss recent results that show conserved architectures and active sites for members of the 5'-nuclease superfamily. Despite substrate diversity, this analysis suggests a common unified mechanism for sequence-independent substrate recognition and incision. The emerging superfamily hallmark is a surprising gating mechanism whereby double nucleotide unpairing of substrates is required to access the active site.

Structure-sensing nucleases

Structure-specific phosphodiesterases (see Glossary) that act without regard to RNA or DNA sequence but in response to formation of aberrant nucleic acid structures are required in all aspects of nucleic acid metabolism [1–4]. During replication or recombination of double-stranded DNA (dsDNA), potentially toxic DNA junctions arise that must be processed [5–9]. Likewise, DNA duplex discontinuities that are formed as a consequence of damage also require the action of structure-specific nucleases [10–14]. Furthermore when DNA is transcribed into RNA, structure-sensing exoribonucleases control transcription termination, processing of non-coding RNAs and the subsequent lifetime of transcripts [15–17]. This structure-based nucleolytic action is critical for life because it maintains nucleic acid information fidelity. However, as inappropriate phosphodiester hydrolysis endangers nucleic acid information content and function, these phosphodiesterases must have high specificity for particular substrate structures and often display tight regulation [18].

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Despite this exquisite specificity requirement, similar protein architectures are paradoxically shared by a superfamily of DNA and RNA processing 5'-nucleases: each somehow specific to particular nucleic acid structures [7,19–23]. The 5'-nuclease superfamily is typified by the essential replication enzyme flap endonuclease (FEN), so is also termed the FEN superfamily (Figure 1). Conservation of 5'-nuclease protein structure presents intriguing questions of selectivity and mechanism. How are individual family members specific for their substrates in the context of a common protein fold? In each case how are specific phosphate diester bonds selected for hydrolysis? Recent combined structural and functional analyses unveil the unexpected mechanism for selective, sequence-independent nucleolytic activity with consequences for understanding the biology of nucleic acid processes [22–30]. Here we review and integrate the lessons learnt from current analyses of FEN superfamily members that act upon substrates during DNA replication and repair, and then extend them to other family members where structural information is incomplete. Furthermore, we draw new analogies between the 5'-nucleases of DNA and RNA metabolism and argue that collective data suggests that these too share FEN superfamily mechanisms.

Roles and substrate specificity of DNA 5'-nucleases

Activities of 5'-nuclease superfamily members span all areas of DNA metabolism. Consequently, substrate specificities appear extraordinarily diverse (Figure 2a). The prototypical superfamily member, flap endonuclease (FEN), catalyzes the endonucleolytic removal of DNA and RNA 5'-flaps from nucleic acid junctions that are generated during strand displacement DNA synthesis [5,6,9,31–33]. FEN proteins are essential for the removal of RNA primers during Okazaki fragment maturation and are also required for long patch base excision repair. FENs enhance the rate of hydrolysis of specific phosphodiester bonds by a factor of at least 10^{17} [34–36]. *In vitro* rates of reaction are equivalent to those of encounter of enzyme and substrate [37,38]. So FEN proteins catalyze reactions at the maximal rate possible for any enzyme reaction. During lagging strand DNA synthesis, PCNA (proliferating cell nuclear antigen) coordinates the interactions of DNA polymerase, FEN1 and DNA ligase [39]; however, FEN1 catalytic activity is not drastically altered by PCNA.

Exonuclease-1 (EXO1) is a close FEN1 paralogue that is found in eukaryotes. Essential for genome maintenance, EXO1 carries out processive 5'-exonucleolytic reactions on nicks, gaps and blunt DNAs, which are formed during mismatch repair and double-strand break repair [13,14,22,40] (Figure 2a). The catalytic efficiency of C-terminally truncated EXO1 variants resemble those of FEN proteins [21]. It is suggested that full length EXO1 contains inhibitory domains allowing regulation by interactions with other proteins [22]. Like FENs, EXO1 can catalyze endonucleolytic flap removal [21]. Similarly, when presented with DNA ends lacking 5'-flaps, FEN proteins act as 5'-exonucleases [36,38,41].

Other FEN superfamily proteins act *in vivo* on substrates that lack 5'-termini (Figure 2a). XPG is the indispensable 5'-nuclease of general genome and transcription coupled nucleotide excision repair [12,42–46]. XPG is a component of the transcription factor IIIH complex, and targets DNA bubbles. Furthermore, GEN1 (flap endonuclease GEN homolog 1), which is proposed to be the major Holliday junction (HJ) resolvase in humans, recognizes four-way mobile DNA junctions that are generated during homologous recombination [7,47–49].

Below, we show how a conserved protein architecture, found in FEN superfamily enzymes, accommodates a diverse range of substrate topologies yet retains specificities (Figure 2a). We discuss how some superfamily members can act on substrates with 5'-ends, whereas others do not. Two key observations provide an initial clue to this paradoxical selectivity: in

all substrates the reacting (“incised”) strand is in the form of a duplex, albeit contained within the context of a more complex DNA junction structure (Figure 2a). Moreover, the major site of reaction in all substrates is one nucleotide into this double-strand.

FEN superfamily proteins are junction resolvases

Structures of the human 5′-nucleases FEN1 (hFEN1) and EXO1 (hEXO1), together with bacteriophage homologues (T5FEN and T4FEN (formerly referred to as T4RNaseH)), show core protein architectures with conserved DNA-binding and catalytic motifs (Figure 1 and Supplemental figure 1) [22–25,50]. Later additions to the ancient 5′-nuclease core seen in bacteriophage FENs produce a diverse superfamily by C-terminal extensions (FEN1, EXO1, GEN1, XPG) or inserts (XPG) (Figure 1a). These additional domains act to specialize function (FEN1) or to regulate and/or coordinate activity with other proteins through protein-protein interactions (FEN1, EXO1, GEN1, XPG) (Figure 1) [51,52]. The presence of two separate nucleic acid binding sites require substrates to sharply bend 90–100° at their dsDNA-dsDNA or dsDNA-ssDNA interfaces, which accounts for the overall specificity of DNA 5′-nucleases for resolving aberrant nucleic acid junctions. Enzyme-substrate interactions occur mainly with the complementary DNA strand and are spaced a helical turn apart [22–24] (Figure 2b). As a consequence of complementary strand binding and bending, the reaction site at the 5′-end of the duplex is directed toward the superfamily conserved active site.

The reacting duplex binding site

The duplex to undergo reaction is bound by a non-specific dsDNA interaction motif, the helix-two/three-turn-helix (H2/3TH), also found in other DNA repair proteins and polymerases (Figures 1b, 3a–b) [1,3,53,54]. This motif binds a potassium ion that interacts with the complementary strand backbone, which may facilitate processivity in some family members. Basic residues from the H2/3TH motif also contact the complementary DNA strand. An interaction motif that resembles a β-pin provides a basic residue to make one of few interactions to the reacting strand as it passes underneath the complementary strand towards a wedging helix. This helix stacks on the terminal base pairs of the duplex. The features of this binding site are largely conserved across the superfamily. In T4FEN [24,30], but not in its close homologue T5FEN [25], a slightly different arrangement of a helix-turn-helix motif, with an insert and no bound K⁺, leads to alternative contacts albeit to the same region of the duplex (Figures 1, 2b).

The secondary DNA binding site

Some substrate selectivity among FEN superfamily members comes from the secondary nucleic acid binding site that variously accommodates ssDNA or dsDNA (Figures 1, 2a, 3a–d). The wedging helix and the loop extending from this helix enforce complementary strand bending, allowing the hairpin of the β-pin to contact the substrate. In T4FEN, which has a preference for ssDNA in this site, aromatic and hydrophobic residues interact with nucleobases. For higher evolutionary FEN1 proteins, cellular substrates are 5′-3′-double flaps (Figure 2a) [36,41,55–57]. The C-terminal region of FEN1 proteins provides a helix-hairpin-helix motif to the secondary site, which is absent from the hEXO1 and bacteriophage FEN structures (Figure 3a–d). Together with the wedge helix loop, this helix-hairpin-helix motif forms a substrate induced binding pocket that cradles a single nucleotide unpaired 3′-flap [23,29] (Figure 3a). In eukaryotic FEN1 proteins, a cluster of acidic residues prevent the 3′-flap being longer than one nucleotide [23]; this “acid block” produces high selectivity for a particular 5′-3′-double flap conformer of the equilibrating products of DNA strand displacement synthesis. Hence, hFEN1 catalyzed incision of double flaps takes place one nucleotide into the reacting duplex to produce only nicked DNAs.

Consequently, the efficiency and fidelity of replication are ensured, as nicked DNA products can directly undergo ligation.

Double nucleotide unpairing

Like most nucleases, FEN superfamily members are metalloenzymes whose active sites coordinate essential divalent metal ions [2,58]. The metal ions must intimately contact the phosphodiester targeted for reaction (the “scissile phosphodiester bond”) to catalyze hydrolysis [58]. Substrate junction binding and bending by FEN family proteins places the scissile phosphodiester near the active site, but not yet positioned on metal ions to undergo hydrolysis (Figure 4a–b). However, in product structures the cleaved 5′-phosphate monoester is directly (inner-sphere) coordinated by two active site metal ions less than 4 Å apart, in accord with functional data consistent with a hydrolytic mechanism involving two ions [59,60] (Figures 3e–f, 4a–b). To achieve this state, the terminal nucleotide of the product is unpaired, implying that a substrate positioned for reaction must have two unpaired nucleotides (Figure 4a). As the characteristic reaction of all 5′-nucleases occurs one nucleotide into the duplex, double nucleotide unpairing is the unexpected hallmark of the FEN superfamily [20,22,23,59].

Redefinition of the 5′-nuclease active site

The original identification of 5′-nucleases highlighted seven carboxylate- residues that were equivalently positioned and seemed to constitute the active sites (Supplemental Figure 1). However, crucial roles for other superfamily-conserved residues are now evident (Figures 3e–f, 4). Invariant lysine and arginine (Lys93 and Arg100 in hFEN1) residues from α -helix 4 (α 4) make contacts with the 5′-phosphate of the unpaired product; mutating either residue drastically impairs reaction [22,23,38,61,62]. To allow the scissile bond contact with active site metals, the adjacent phosphate diester has to traverse the carboxylate rich active site. To stabilize this, the phosphate diester interacts with the N-terminal residue of the protein (Gly2, the result of co-translational removal of Met1). In bacteriophage enzymes, where the N-terminus is a site of protein-protein interaction, an extra metal ion plays a similar role (Figure 4d) [60]. In the hFEN1-product structure, the unpaired 5′-terminal nucleobase stacks with a tyrosine residue from the wedge helix. Before unpairing, the same tyrosine interacts with the 5′-terminal nucleobase of duplex that has departed in the product complex (Figure 4a–c). In hEXO1, a histidine residue stacks on the paired 5′-nucleobase initially (Figure 4e). Thus, besides the seven carboxylates that coordinate at least two divalent metal ions, the 5′-nuclease active site can be redefined to also include conserved α 4 lysine and arginine residues, either the N-terminus or a third metal ion, and stacking partner(s) from the wedging helix (Figure 4c–g).

A protein gateway to the active site selects ends that can readily unpair

Besides presenting residues to the active site, the conserved protein architecture links substrate selection to catalysis. When ordered and positioned to provide catalytic residues, the base of the wedging helix and the base of α 4 form a helical gateway through which only ssDNA can pass (Figure 4). This has two key consequences. First, continuous duplex is protected from the action of 5′-nucleases. Second, the gateway forms a recognition motif for ends of duplexes with an inherent propensity to unpair [63,64], even when contained within more complex structures. Comparisons of hFEN1 complexes with paired and unpaired DNAs suggest that unpairing may be facilitated by partially unwinding the duplex near the active site [23].

Selectivity for 5'-termini

Although all FEN superfamily 5'-nucleases catalyze reactions of the ends of duplexes at nucleic acid junctions, not all select for 5'-termini like FEN and EXO1 proteins do. How this selection occurs is controversial. Unlike other superfamily members, protein interaction partners do not tightly regulate FEN activity, so FENs must have intrinsic specificity features [52]. FEN proteins must exclude potential substrates that lack free 5'-ends to avoid damaging genome integrity at the replication fork. This was proposed to be controlled by the helical cap that extends from gateway $\alpha 4$, under which 5'-flaps could thread exiting from the back of the protein [6,23,25] (Figures 1, 3e–f). The cap is not preserved in members such as XPG and GEN1 that do not select for free 5'-termini [23]. The cap and co-joined gateway $\alpha 4$ are (partially) disordered in many FEN structures, such as that of hFEN1 in the absence of substrate [50]. When ordered, the cap creates a hole through which ssDNA with a free 5'-terminus but not dsDNA can pass.

One model to explain FEN specificity, known as the tracking model, postulated that FEN proteins initially interacted with single-stranded flaps and then slid down them until a ssDNA-dsDNA junction was encountered [65]. However, the tracking model was ruled out by the observations that the main DNA binding interface does not include 5'-flaps, that exonucleolytic and endonucleolytic reactions display comparable efficiencies, and that FEN1 possesses the ability to process gapped flaps (Figure 2a) [22,23,36,66]. A second model proposed that complementary strand junction binds first, and then flap threading takes place. This model is consistent with the known protein structures, but raises concerns about how to push and pull the ssDNA through a small hole, and cannot account for the ability of FEN proteins to incise gapped flaps [36,67]. In a third clamping model, flaps exit from the active site at the front of the protein past $\alpha 4$ but not under the cap (in the cases of FEN and EXO1), or substrate topology dictates that 5'-parts of substrate travel between the wedging helix and the equivalent of $\alpha 5$ (in the cases of XPG and GEN1) [22]. However, the clamping model is difficult to reconcile with a need to enforce specificity for free 5'-ends.

We favour an alternative model, consistent with the structural changes observed upon formation of ternary complexes, whereby 5'-flaps thread through a disordered aperture that subsequently orders to position the cap and gateway to effect reaction (Figure 3e,f, 5a) [23]. Notably, this disorder-thread-order mechanism would permit regions of flap duplex (gapped flaps, Figure 2a) and flaps with moderately bulky modifications to pass through the aperture, but would exclude flaps with larger modifications (e.g. bound protein) or substrates that lack 5'-termini [36,65,66,68]. With the cap and gateway ordered and the unpaired substrate in the active site, only the first unpaired nucleotide located 5' of the scissile phosphodiester would be enclosed within the gateway under the cap (i.e., the 5'-nucleotide of exonucleolytic substrates). Support for this hypothesis comes from the T4FEN-DNA structure without active site metals (Figure 1b), in which a 5'-flap departs as though it passes under the cap. Without metals the substrate cannot position for reaction, so the cap remains partially disordered.

Flexible caps, gateways and wedges confer selectivity

Whilst the basis for selectivity for 5'-termini remains controversial, there is agreement on the potential of helical gateway conformation to control reactivity by altering the position of the critical active site lysine and arginine residues. It has been suggested that larger members of the FEN superfamily are regulated by protein partners that interact directly with the cap and gateway, or with the C-terminal domain, which is seen in truncated form at the back of the cap and gateway in hEXO1 structures (Figure 3d–f) [22]. It is also posited that hFEN1 uses disorder-to-order equilibria to confer selectivity for double flap substrates [23]. The

part of the C-terminal region that is closest to the main body of the protein participates in the formation of a 3'-flap binding site. It is thought that substrate binding results in ordering of the 3'-flap binding pocket. As a result of wedge-cap interactions, the ordering of the 3'-flap binding pocket facilitates cap ordering (Figure 3a). We propose that the release of the bound 5'-phosphorylated product requires a conformational change in the protein, and that gateway positioning (influenced by protein and substrate structure) may act in catalysis for all family members. Rounds of disorder-order-disorder transitions would allow processive cleavage of substrates, which is prevented in FEN1 proteins by the 3'-flap pocket.

Extending lessons learned from FEN1 and EXO1 to XPG and GEN1

Whilst there are no available structures of XPG and GEN1, conservation of the FEN superfamily active site and gateway implies that these proteins use double nucleotide unpairing mechanisms. DNA structures processed by XPG and GEN1 are reminiscent of the FEN1 double flap; a bubble is a covalently linked double flap substrate, and a HJ is a fused double flap (Figure 2a). Accommodation of these substrates is plausible, as the cap that would prevent such substrates in FENs and EXO1 is not conserved in XPG or GEN. Removal of the cap would allow a large 30 nucleotide DNA bubble access to the XPG active site and enable binding to the catalytic core (Figure 5b). For GEN, which incises two strands of the Holliday junction, we suggest that a dimer of the catalytic core, with one monomer rotationally related to the other and with the active sites facing each other, provides an elegant model consistent with a double incision, which would require only a short ssDNA at the HJ center (Supplemental Movie). For efficient binding to the substrate, protein dimerization may occur after one subunit binds to the substrate, or the HJ may be threaded between the dimers while these are stretched out from each other by a disordered gateway. Formation of a dimer-substrate complex would then be the trigger for a disorder-order transition of the gateway. Biologically, the two incised strands would leave the dimer on the same side, with implications in positioning of other HJ pathway proteins.

Do RNA 5'-nucleases conserve FEN-like mechanisms?

Earlier analyses noted sequence similarities between FEN proteins and the 5'-3' exoribonucleases (XRN) that play crucial roles in transcription termination, RNA turnover and RNA interference ultimately controlling gene expression [15–17,69] (Supplemental figure 1). XRN target 5'-monophosphorylated RNAs for processive hydrolysis to single nucleotide products [70]. Xrn2 (known as Rat1 in yeast) functions primarily in the nucleus, whereas Xrn1 is its cytosolic counterpart. Unlike other FEN superfamily members, XRN do not target particular junctions in their substrates. Rather, concomitant with their degradation role, XRN must deal with all types of RNA secondary structure including intramolecular duplex. The structure sensed by XRN is the status of the 5'-terminus of substrates; XRN only act when a protective m⁷GpppN-cap is removed to target RNAs for destruction. Despite this apparent difference with other 5'-nucleases recent structural studies of SpXrn2 (from *Schizosaccharomyces pombe*) and KIXrn1 (from *Kluyveromyces lactis*), together with a complex of DmXrn1 (from *Drosophila melanogaster*) with a short fragment of DNA, reveal striking similarities to DNA nucleases of the FEN superfamily (Figures 4f–g, 5c) [26–28].

Whilst XRN are much larger than FENs, they conserve the nucleic acid binding motifs (H2/3TH and β -pin) and the characteristic active site of the FEN superfamily, including all seven carboxylates and the helical gateway with associated basic and stacking residues (Figures 4f–g, 5c). The XRN N-terminus is within the active site, but retains Met1 in the DmXrn1 structure and residues resulting from cloning procedures in SpXrn2 [26,28]. A helical cap, in varying states of disorder, is also present in the XRN [26–28] (Figure 5c).

Reminiscent of the C-terminal region of the hEXO1, the large XRN C-terminus extends behind the protein and wraps around the FEN-like nuclease domain. Conspicuously, in all current XRN structures the C-terminus would block exit of threaded flap substrates from the back of the cap and gateway. Thus, in the DmXrn1-DNA structure the 5'-nucleotide of ssDNA is bound in a pocket formed from the two gateway helices. Analogous to FEN superfamily structures, the nucleobase is stacked on a histidine from the wedging helix and the 5'-phosphate is contacted by the DNA superfamily conserved arginine [26].

Although the DmXrn1-DNA scissile phosphodiester is not positioned on the active site metal, the substrate penetrates further through the gateway than the 5'-phosphorylated product DNAs in the structures of hFEN1 and hEXO1; thus, the DmXrn1-DNA structure resembles a threaded complex (Figure 4f). By analogy to DNA 5'-nucleases (Figures 4a, 5a), movement of the scissile bond down to the active site metals could require ordering of the helical cap and transfer of the histidine stacking partner to the penultimate nucleobase. Whether this would furnish a threaded structure or require a sharp turn to provide a clamped substrate remains debatable. Yet, the observed 5'-phosphate binding mode, where larger 5'-structures are excluded by the C-terminal domain, does explain why m⁷GpppN-capped or 5'-triphosphorylated RNAs are refractory to hydrolysis by XRN, and why RNA 5'-flaps are processed to single nucleotide products and not endonucleolytic products. This selectivity would be more difficult to reconcile with a clamped structure. Although an alternative model for translocation-coupled unwinding of structured RNA substrates by the XRN has been postulated [26], we suggest that the extraordinary conservation of 5'-nuclease superfamily features advocates double nucleotide unpairing and disorder-order transition mechanisms analogous to those discovered in DNA 5'-nucleases (Figures 4a, 5a).

Concluding remarks and future directions

The existence of FENs in bacteriophages, bacteria, archaea and eukaryotes and further evolution of other superfamily paralogs, indicates that an ancient enzyme activity essential to life is employed throughout nucleic acid metabolism [71]. Together, analyses of FEN superfamily members allow us to see an integrated picture that is adapted by each 5'-nuclease in turn to provide its unique specificity features (Figure 5a,b). As exemplified for FEN1, the initial binding of dsDNA to the H2/3TH region is followed by bending of the malleable junction and the binding of the 3'-flap. This directs the 5'-flap through and under the disordered helical gateway and cap (Figure 5a). A subsequent disorder-to-order transition and double nucleotide unpairing, permits two-metal ion incision. Double nucleotide unpairing explains both the reaction site specificity of 5'-nucleases and the ease by which exo- and endonucleolytic activities can be accommodated. Variation in the helical cap and the secondary binding site dictate diverse specificities whilst providing junction specificity for DNA flaps, free ends, bubbles, and HJs (Figure 5b). These conserved binding and catalytic elements reveal the design principles of a dynamic nucleic acid cutting machine that can be specific for DNA, RNA, or hybrid junctions.

Just as nucleotide flipping provides selection for sites of base damage in dsDNA [1], gating and double nucleotide unpairing provide an elegant mechanism whereby nucleases can selectively allow ends of junctions, but not other nucleic acid structures, to access the active site. To date, FEN superfamily members are associated with DNA replication, every major DNA repair pathway, and likely RNA metabolic processes in the nucleus and cytoplasm. Other 5'-nucleases may possibly exist where DNA or RNA junctions need processing; some evidence for this exists in bacteria [72]. Conservation in architecture and activity, aided by the redefined active site, provides the basis to identify possible new superfamily members (Figures 1,4,5). Yet, double base unpairing will also limit potential 5'-nuclease activities. For example, FEN-superfamily members will not be able to process interstrand crosslinked

DNA lesions; this explains the need for structure-specific endonucleases that do not belong to the FEN superfamily for the Fanconi anemia DNA damage response network [73].

Due to their essential replication and repair activities, FEN superfamily proteins have key roles in cell proliferation, fidelity and cancer risk [52,74] and therefore are potential targets for therapeutic intervention. This is exemplified by specific synthetic lethal killing of RAD54B-deficient human colorectal cancer cells by FEN1 silencing [75]. At replication forks, the activities of FEN1, Mre11, EXO1, or XPG on the same DNA substrates control different pathways and outcomes; for example, GEN1 can control HJ formation depending upon DNA interactions with various proteins such as PCNA, RAD50, BRCA2, Ku, or WRN [76–78]. A better understanding of the underlying enzymatic mechanisms offers the promise of specific 5′-nuclease inhibitors [79]. FEN1 is also a powerful tool for invasive signal amplification reactions [80] to form sensitive detection assays for DNA and RNA [81]. New understandings of the processing of nucleic acid junctions by 5′-nucleases, therefore, have broad implications for cellular and molecular biology as well as medicine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Glossary

DNA bubble	Duplex DNA that contains a central unpaired region formed during replication, transcription, and repair
Endonucleolytic reaction	The hydrolysis of a phosphate diester that takes place more than one nucleotide away from either end of a nucleic acid
Exonucleolytic reaction	The hydrolysis of a phosphate diester that takes place at the terminus of a nucleic acid
Fanconi anemia DNA damage response network	A network of proteins devoted to responding to modification of DNA by interstrand crosslinks
Holliday junction	A mobile four-way junction that consists of four DNA strands forming four dsDNA segments linked together
Homologous recombination	Exchange of DNA between homologous DNA that occurs naturally during meiosis or DNA repair
Long patch base excision repair	A pathway that removes damaged nucleotide bases from DNA by glycolytic action and is responsible for the removal of 5′-deoxyribosyl moieties
Lagging strand DNA synthesis	The replication machinery forms a fork, with one strand synthesized continuously (leading strand) and the other strand (lagging strand) replicated in short segments known as Okazaki fragments

Mismatch repair	Pathway for the removal of nucleotide mismatches that are incorporated by polymerases or that result from base deamination
Non-Coding RNA	Transcribed gene whose RNA is not translated, but performs regulatory roles or act as a guide during RNA processing
Nucleic acid junction	A branchpoint between double helical segments and/or single stranded regions in folded RNA or DNA structures
Nucleotide excision repair	Pathway for the removal and replacement of bulky DNA lesions
Okazaki fragment	Short DNA segment that is generated on the lagging strand during DNA replication due to continuous re-priming of newly exposed DNA at the replication fork
Okazaki fragment maturation	Lagging-strand DNA replication process whereby Okazaki fragments are converted into a continuous piece of DNA through a multi-step process
Phosphodiesterase	Enzyme that catalyzes the hydrolysis of phosphate diester bonds, also known as nuclease when nucleic acid phosphate diester bonds are targeted
Resolvase	A phosphodiesterase targeting a DNA junction with the eventual aim of restoring duplex DNA
RNA interference	Control of gene expression through by the introduction of foreign RNA or by the expression of a microRNA that results in targeted mRNA degradation or the inhibition of mRNA translation
RNA turnover	Process whereby an mRNA is degraded to control gene expression, remove damaged or mRNAs containing premature stop codons
Strand displacement DNA synthesis	Process whereby a polymerase initially incorporates nucleotides in a ssDNA region but, upon reaching the adjacent dsDNA segment, continues DNA synthesis by displacing the complementary strand, creating a nucleic acid junction known as a 5'-flap
Synthetic lethal	A combination of deletions, mutations, knock-downs, or combination thereof in two or more genes that leads to cell death

References

1. Hitomi K, et al. The intricate structural chemistry of base excision repair machinery: implications for DNA damage recognition, removal, and repair. *DNA Repair (Amst)*. 2007; 6:410–428. [PubMed: 17208522]
2. Yang W. Nucleases: diversity of structure, function and mechanism. *Quart Rev Biophys*. 2011; 44:1–93.
3. Huffman J, et al. DNA base damage recognition and removal: New twists and grooves. *Mutation Research*. 2005; 577:55–78. [PubMed: 15941573]
4. Williams R, Kunkel T. FEN Nucleases: Bind, Bend, Fray, Cut. *Cell*. 2011; 145:171–172. [PubMed: 21496637]

5. Harrington JJ, Lieber MR. The characterization of a mammalian DNA structure-specific endonuclease. *EMBO J.* 1994; 13:1235–1246. [PubMed: 8131753]
6. Lyamichev V, et al. Structure-specific endonucleolytic cleavage of nucleic acids by eubacterial DNA polymerases. *Science.* 1993; 260:778–783. [PubMed: 7683443]
7. Ip SCY, et al. Identification of Holliday junction resolvases from humans and yeast. *Nature.* 2008; 456:357–361. [PubMed: 19020614]
8. Zheng L, Shen B. Okazaki fragment maturation: nucleases take centre stage. *Journal of Molecular Cell Biology.* 2011; 3:23–30. [PubMed: 21278448]
9. Wu XT, et al. Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA. *Nucleic Acids Res.* 1996; 24:2036–2043. [PubMed: 8668533]
10. Klungland A, Lindahl T. Second pathway for completion of human DNA base excision-repair: reconstitution with purified proteins and requirement for DNase IV (FEN1). *EMBO J.* 1997; 16:3341–3348. [PubMed: 9214649]
11. Aboussekhra A, et al. Mammalian DNA nucleotide excision-repair reconstituted with purified protein-components. *Cell.* 1995; 80:859–868. [PubMed: 7697716]
12. Odonovan A, et al. XPG Endonuclease makes the 3'-incision in human DNA nucleotide excision-repair. *Nature.* 1994; 371:432–435. [PubMed: 8090225]
13. Genschel J, et al. Human exonuclease I is required for 5' and 3' mismatch repair. *J Biol Chem.* 2002; 277:13302–13311. [PubMed: 11809771]
14. Zhu Z, et al. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. *Cell.* 2008; 134:981–994. [PubMed: 18805091]
15. Kim M, et al. The yeast Rat1 exonuclease promotes transcription termination by RNA polymerase II. *Nature.* 2004; 432:517–522. [PubMed: 15565157]
16. Fabian MR, et al. Regulation of mRNA Translation and Stability by microRNAs. *Annual Review of Biochemistry.* 2010; 79:351–379.
17. Garneau NL, et al. The highways and byways of mRNA decay. *Nature Reviews Molecular Cell Biology.* 2007; 8:113–126.
18. Ciccia A, Elledge SJ. The DNA Damage Response: Making It Safe to Play with Knives. *Mol Cell.* 2010; 40:179–204. [PubMed: 20965415]
19. Lieber MR. The FEN-1 family of structure-specific nucleases in eukaryotic DNA replication, recombination and repair. *Bioessays.* 1997; 19:233–240. [PubMed: 9080773]
20. Tomlinson CG, et al. Substrate Recognition and Catalysis by Flap Endonucleases and Related Enzymes. *Biochem Soc Trans.* 2010; 38:433–437. [PubMed: 20298197]
21. Lee BI, Wilson DM. The RAD2 domain of human exonuclease 1 exhibits 5' to 3' exonuclease and flap structure-specific endonuclease activities. *J Biol Chem.* 1999; 274:37763–37769. [PubMed: 10608837]
22. Orans J, et al. Structures of Human Exonuclease 1 DNA Complexes Suggest a Unified Mechanism for Nuclease Family. *Cell.* 2011; 145:212–223. [PubMed: 21496642]
23. Tsutakawa SE, et al. Human Flap Endonuclease Structures, DNA Double Base Flipping and a Unified Understanding of the FEN1 Superfamily. *Cell.* 2011; 145:196–211.
24. Devos JM, et al. Crystal structure of bacteriophage T4 5' nuclease in complex with a branched DNA reveals how FEN-1 family nucleases bind their substrates. *J Biol Chem.* 2007; 282:31713–31724. [PubMed: 17693399]
25. Ceska TA, et al. A helical arch allowing single-stranded DNA to thread through T5 5'-exonuclease. *Nature.* 1996; 382:90–93. [PubMed: 8657312]
26. Jinek M, et al. Coupled 5' Nucleotide Recognition and Processivity in Xrn1-Mediated mRNA Decay. *Mol Cell.* 2011; 41:600–608. [PubMed: 21362555]
27. Chang JH, et al. Structural and biochemical studies of the 5' → 3' exoribonuclease Xrn1. *Nat Struct Mol Biol.* 2011; 18:270–276.
28. Xiang S, et al. Structure and function of the 5' → 3' exoribonuclease Rat1 and its activating partner Rai1. *Nature.* 2009; 458:784–788. [PubMed: 19194460]
29. Chapados BR, et al. Structural basis for FEN-1 substrate specificity and PCNA-mediated activation in DNA replication and repair. *Cell.* 2004; 116:39–50. [PubMed: 14718165]

30. Mueser TC, et al. Structure of bacteriophage T4 RNase H, a 5' to 3' RNA-DNA and DNA-DNA exonuclease with sequence similarity to the Rad2 family of eukaryotic proteins. *Cell*. 1996; 85:1101–1112. [PubMed: 8674116]
31. Liu Y, et al. Flap endonuclease 1: a central component of DNA metabolism. *Annu Rev Biochem*. 2004; 73:589–615. [PubMed: 15189154]
32. Larsen E, et al. Proliferation failure and gamma radiation sensitivity of Fen1 null mutant mice at the blastocyst stage. *Mol Cell Biol*. 2003; 23:5346–5353. [PubMed: 12861020]
33. Kucherlapati M, et al. Haploinsufficiency of flap endonuclease (FEN-1) leads to rapid tumour progression. *Proc Natl Acad Sci U S A*. 2002; 99:9924–9929. [PubMed: 12119409]
34. Pickering TJ, et al. A single cleavage assay for T5 5'→3' exonuclease: determination of the catalytic parameters for wild-type and mutant proteins. *Nucleic Acids Res*. 1999; 27:730–735. [PubMed: 9889266]
35. Tock MR, et al. Dynamic evidence for metal ion catalysis in the reaction mediated by a flap endonuclease. *EMBO J*. 2003; 22:995–1004. [PubMed: 12606565]
36. Finger LD, et al. The 3'-flap pocket of human flap endonuclease 1 is critical for substrate binding and catalysis. *J Biol Chem*. 2009; 284:22184–22194. [PubMed: 19525235]
37. Finger LD, Shen B. FEN1 (flap structure-specific endonuclease 1). *Atlas Genet Cytogenet Oncol Haematol*. 2010
38. Sengerová B, et al. Brønsted Analysis and Rate-Limiting Steps for the T5 Flap Endonuclease Catalyzed Hydrolysis of Exonucleolytic Substrates. *Biochemistry*. 2010; 49:8085–8093. [PubMed: 20698567]
39. Kunkel TA, Burgers PM. Dividing the workload at a eukaryotic replication fork. *Trends in Cell Biology*. 2008; 18:521–527. [PubMed: 18824354]
40. Wei KC, et al. Inactivation of Exonuclease 1 in mice results in DNA mismatch repair defects, increased cancer susceptibility, and male and female sterility. *Genes & Dev*. 2003; 17:603–614. [PubMed: 12629043]
41. Williams R, et al. Comparison of the catalytic parameters and reaction specificities of a phage and an archaeal flap endonuclease. *J Mol Biol*. 2007; 371:34–48. [PubMed: 17559871]
42. Hohl M, et al. Structural Determinants for Substrate Binding and Catalysis by the structure specific endonuclease XPG. *J Biol Chem*. 2003; 278:19500–19508. [PubMed: 12644470]
43. Staresinic L, et al. Coordination of dual incision and repair synthesis in human nucleotide excision repair. *EMBO J*. 2009; 28:1111–1120. [PubMed: 19279666]
44. Sarker AH, et al. Recognition of RNA Polymerase II and Transcription Bubbles by XPG, CSB, and TFIIH: Insights for transcription-coupled repair and cockayne syndrome. *Mol Cell*. 2005; 20:187–198. [PubMed: 16246722]
45. Ito S, et al. XPG stabilizes TFIIH, allowing transactivation of nuclear receptors: Implications for Cockayne syndrome in XP-G/CS patients. *Molecular Cell*. 2007; 26:231–243. [PubMed: 17466625]
46. Fuss J, Tainer J. XPB and XPD helicases in TFIIH orchestrate DNA duplex opening and damage verification to coordinate repair with transcription and cell cycle via CAK kinase. *DNA repair*. 2011; 10:697–713. [PubMed: 21571596]
47. Rass U, et al. Mechanism of Holliday junction resolution by the human GEN1 protein. *Genes & Dev*. 2010; 24:1559–1569. [PubMed: 20634321]
48. West SC. The search for a human Holliday junction resolvase. *Biochem Soc Trans*. 2009; 37:519–526. [PubMed: 19442245]
49. Bailly AP, et al. The *Caenorhabditis elegans* Homolog of Gen1/Yen1 Resolvases Links DNA Damage Signaling to DNA Double-Strand Break Repair. *Plos Genetics*. 2010; 6.
50. Sakurai S, et al. Structural basis for recruitment of human flap endonuclease 1 to PCNA. *EMBO J*. 2005; 24:683–693. [PubMed: 15616578]
51. Hohl M, et al. Domain swapping between FEN-1 and XPG defines regions in XPG that mediate nucleotide excision repair activity and substrate specificity. *Nucleic Acids Res*. 2007; 35:3053–3063. [PubMed: 17452369]

52. Zheng L, et al. Functional regulation of FEN1 nuclease and its link to cancer. *Nucleic Acids Res.* 2011; 39:781–794. [PubMed: 20929870]
53. Pelletier H, Sawaya MR. Characterization of the metal ion binding helix-hairpin-helix motifs in human DNA polymerase beta by X-ray structural analysis. *Biochemistry.* 1996; 35:12778–12787. [PubMed: 8841120]
54. Thayer M, et al. Novel DNA binding motifs in the DNA-repair enzyme endonuclease III structure. *EMBO J.* 1995; 14:4108–4120.
55. Kaiser MW, et al. A comparison of eubacterial and archaeal structure-specific 5'-exonucleases. *J Biol Chem.* 1999; 274:21387–21394. [PubMed: 10409700]
56. Friedrich-Heineken E, Hubscher U. The Fen1 extrahelical 3'-flap pocket is conserved from archaea to human and regulates DNA substrate specificity. *Nucleic Acids Res.* 2004; 32:2520–2528. [PubMed: 15131255]
57. Kao KI, et al. Cleavage specificity of *Saccharomyces cerevisiae* flap endonuclease 1 suggests a double-flap structure as the cellular substrate. *J Biol Chem.* 2002; 277:14379–14389. [PubMed: 11825897]
58. Yang W, et al. Making and breaking nucleic acids: Two-Mg²⁺-ion catalysis and substrate specificity. *Mol Cell.* 2006; 22:5–13. [PubMed: 16600865]
59. Syson K, et al. Three metal ions participate in T5 flap endonuclease catalysed DNA hydrolysis. *J Biol Chem.* 2008; 283:28741–28746. [PubMed: 18697748]
60. Tomlinson C, et al. Neutralizing Mutations of Carboxylates That Bind Metal 2 in T5 Flap Endonuclease Result in an Enzyme That Still Requires Two Metal Ions. *J Biol Chem.* 2011; 286:30878–30887. [PubMed: 21734257]
61. Dervan JJ, et al. Interactions of mutant and wild-type flap endonucleases with oligonucleotide substrates suggest an alternative model for DNA binding. *Proc Natl Acad Sci USA.* 2002; 99:8542–8547. [PubMed: 12084915]
62. Storici F, et al. The flexible loop of human FEN1 endonuclease is required for flap cleavage during replication and repair. *EMBO J.* 2002; 21:5930–5942. [PubMed: 12411510]
63. Jose D, et al. Spectroscopic studies of position-specific DNA “breathing” fluctuations at replication forks and primer-template junctions. *Proc Natl Acad Sci U S A.* 2009; 106:4231–4236. [PubMed: 19246398]
64. McKinney SA, et al. Observing spontaneous branch migration of Holliday junctions one step at a time. *Proc Natl Acad Sci U S A.* 2005; 102:5715–5720. [PubMed: 15824311]
65. Murante RS, et al. Calf 5' to 3' exo/endonuclease must slide from a 5' end of the substrate to perform structure-specific cleavage. *J Biol Chem.* 1995; 270:30377–30383. [PubMed: 8530463]
66. Gloor JW, et al. Flap Endonuclease 1 Mechanism Analysis Indicates Flap Base Binding Prior to Threading. *J Biol Chem.* 2011; 285:34922–34931. [PubMed: 20739288]
67. Xu Y, et al. Contacts between the 5' nuclease of DNA polymerase I and its substrate DNA. *J Biol Chem.* 2001; 276:30167–30177. [PubMed: 11349126]
68. Kao HI, et al. On the roles of *Saccharomyces cerevisiae* Dna2p and flap endonuclease 1 in Okazaki fragment processing. *J Biol Chem.* 2004; 279:15014–15024. [PubMed: 14747468]
69. Solinger JA, et al. Active-site mutations in the Xrn1p exoribonuclease of *Saccharomyces cerevisiae* reveal a specific role in meiosis. *Mol Cell Biol.* 1999; 19:5930–5942. [PubMed: 10454540]
70. Stevens A, Poole TL. 5'-Exonuclease-2 of *Saccharomyces-cerevisiae*-Purification and features of ribonuclease activity with comparison to 5'-exonuclease-1. *J Biol Chem.* 1995; 270:16063–16069. [PubMed: 7608167]
71. Shen BH, et al. Flap endonuclease homologs in archaeobacteria exist as independent proteins. *Trends In Biochemical Sciences.* 1998; 23:171–173. [PubMed: 9612080]
72. Allen LM, et al. Active site substitutions delineate distinct classes of eubacterial flap endonuclease. *Biochem J.* 2009; 418:285–292. [PubMed: 19000038]
73. Kratz K, et al. Deficiency of FANCD2-Associated Nuclease KIAA1018/FAN1 Sensitizes Cells to Interstrand Crosslinking Agents. *Cell.* 2010; 142:77–88. [PubMed: 20603016]

74. Zheng L, et al. Fen1 mutations result in autoimmunity, chronic inflammation and cancers. *Nature Medicine*. 2007; 13:812–818.
75. McManus KJ, et al. Specific synthetic lethal killing of RAD54B-deficient human colorectal cancer cells by FEN1 silencing. *Proceedings Of the National Academy Of Sciences Of the United States Of America*. 2009; 106:3276–3281. [PubMed: 19218431]
76. Schlacher K, et al. Double-Strand Break Repair-Independent Role for BRCA2 in Blocking Stalled Replication Fork Degradation by MRE11. *Cell*. 2011; 145:529–542. [PubMed: 21565612]
77. Trego K, et al. The DNA repair endonuclease XPG interacts directly and functionally with the WRN helicase defective in Werner syndrome. *Cell Cycle*. 2011; 10:1998–2007. [PubMed: 21558802]
78. Williams RS, et al. Mre11 dimers coordinate DNA end bridging and nuclease processing in double-strand-break repair. *Cell*. 2008; 135:97–109. [PubMed: 18854158]
79. Tumey LN, et al. The identification and optimization of a N-hydroxy urea series of flap endonuclease 1 inhibitors. *Bioorg Med Chem Lett*. 2005; 15:277–281. [PubMed: 15603939]
80. Lyamichev VI, et al. Experimental and theoretical analysis of the invasive signal amplification reaction. *Biochemistry*. 2000; 39:9523–9532. [PubMed: 10924149]
81. Allawi HT, et al. Invader plus method detects herpes simplex virus in cerebrospinal fluid and simultaneously differentiates types 1 and 2. *Journal of Clinical Microbiology*. 2006; 44:3443–3447. [PubMed: 16954297]
82. Pei J, et al. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Research*. 2008; 36:2295–2900. [PubMed: 18287115]
83. Gouet P, et al. ESPript: analysis of multiple sequence alignments in PostScript. *Bioinformatics*. 1999; 15:305–308. [PubMed: 10320398]

Figure 1. FEN superfamily nuclease domains have a conserved architecture

(a) Domain alignment of FEN superfamily members that act in DNA metabolic pathways. Schematic shows the relative positions of key motifs and structural elements common among the catalytic cores of FEN superfamily members. Position determined by sequence alignment (Supplemental Figure 1). **(b)** Comparison of FEN superfamily structures. Structures of hFEN1 (residues 2–336) with DNA and active site metals (3Q8K.pdb), hEXO1 (residues 2–346) with DNA and active site metals (3QEB.pdb), T5FEN with active site metals (1UT5.pdb), T4FEN with DNA and without active site metals (2INH.pdb) and T4FEN without active site metals (3H8J.pdb) are coloured as in (a) to highlight conservation of the catalytic core with FEN1 elements labeled showing how the superfamily recognizes substrates. Interactions are mainly with the complementary DNA strand, which has a 100° bend forced by a helical wedge and the β -pin. The reacting duplex is contacted primarily through a K⁺ coordinated by the H2TH motif and the wedge. A superfamily-conserved gateway guards the active site. In FENs and EXO1 a helical cap tops the gateway, partly disordered in T4FEN with substrate. The active site contains seven spatially conserved carboxylates, basic residues from the gateway, active site metal ions (cyan) and a nucleobase stacking residue from the wedge gateway helix.

Figure 2. All FEN superfamily members bind the reacting duplex DNA

(a) FEN superfamily substrates. Schematic showing FEN superfamily substrates, highlighting the duplex common to all substrates (yellow) that undergoes reaction (arrow). (b) Contacts between FEN superfamily proteins and substrates occur at similar positions enforcing substrate bending. DNA from FEN1 (3Q8K.pdb), EXO1 (3QE9.pdb with divalent ions modelled in as in 3QEB), and T4FEN (2IHN.pdb) shows DNA atoms within 4 Å of the protein (spheres: brown for complementary strand, orange for incised strand, and pink for 3'-flap) with the reacting duplex highlighted (yellow). FEN superfamily members interact mainly with the complementary strand and with the terminal 5'-nucleotides of the strand that undergoes reaction, with FEN1 alone having a significant interaction to the 3'-flap terminal nucleotide. Separation of the two major binding regions on the complementary strand (brown) by ~1 helical turn places the 5'-strand to be incised near the active site.

Figure 3. hFEN1 and hEXO1 structures share the same active site and cap architecture but diverge in the secondary nucleic acid binding site

(a–d) Comparison of hFEN1 and hEXO1 structures highlighting similarities and differences. Surface front and back views of (a,c) hFEN1 product (3Q8K.pdb) complex and (b) front view hEXO1 substrate (3QEA.pdb) and (d) back hEXO1 substrate product (3QEB.pdb with K⁺ modelled in based on 3QEA) with proteins as surfaces to highlight substrate-specific binding and divergence in C-terminal regions (gray). Colouring is as figure 1. hFEN1 forms a 3'-flap binding pocket and added complementary strand (template) contacts, partly formed by the divergent region. This region in hEXO1 remains at the back of the gateway and cap. The exit for substrates threaded under the cap is indicated. (e–f) Structural conservation of the active site residues, cap and gateway region in (e) hFEN1 (3Q8K.pdb) and (f) hEXO1 (3QEB.pdb) product complexes. Close-up view of the back of the helical gateway (blue) and cap (pink), showing conserved carboxylates (red), gateway $\alpha 4$ lysine, arginine and $\alpha 2$

stacking residue (blue) and product 5'-phosphate (orange) bound to active site metals (cyan).

Figure 4. Double nucleotide unpairing is required for substrates to enter the conserved 5'-nuclease superfamily active site

(a) Cartoon of the FEN superfamily double nucleotide unpairing mechanism and gateway that selects ends of duplexes for reaction. The gateway (blue), conserved lysine, arginine and stacking residue and active site carboxylates (pink oval) are depicted. Substrate is initially base-paired (blue-light blue, red-violet) but does not contact active site metals (cyan) in substrate complex. Double nucleotide unpairing allows the scissile phosphate diester to enter the active site and contact active site metals (unpaired complex) resulting in reaction and the formation of product complex. (b) Evidence for double nucleotide unpairing from DNA repositioning in hFEN1 substrate and product complexes. The nucleotides on either side of the scissile phosphate diester (*) are paired (blue-light blue, red-violet) in the hFEN1-substrate complex (3Q8L.pdb) and do not contact active site metals. In hFEN1-product complexes (3Q8K.pdb) the substrate 5'-nucleotide has departed and the product terminal nucleotide (red) is unpaired and inner sphere coordinated to active site metals. A FEN-conserved tyrosine (Y40) stacks with the duplex terminal nucleobase (blue) in the substrate complex but then stacks with the terminal nucleobase of product (red) after reaction. (c–g) Comparison of gateway helices, basic residues, stacking partners and active site carboxylates throughout the 5'-nuclease superfamily implying a common double nucleotide unpairing mechanism. Entrance to the active sites is restricted by the gateway formed by two alpha helices (cylinders) that only allow single-stranded nucleic acid to enter.

For structures with DNA, the terminal bases of the 5'-strand are shown (outlines) to demonstrate that the nucleotides must enter the helical gateway for incision. Key conserved catalytic side chains are shown as sticks. The seven spatially conserved carboxylates directly and indirectly coordinate the divalent metals, whereas strictly conserved basic side chains contact the phosphate monoester product. A residue with a side chain that can stack with the terminal base is always present. In hEXO1, two different residues, His36 and Tyr32, stack with terminal nucleotide in the substrate and product complexes, respectively. Differences arise due to mutation (DmXrn1 D207A) that results in only one bound active site ion or the absence or position of the N-terminus in the active site. In T5FEN a metal is bound in the equivalent position to the N-terminus. (c) hFEN1 (3Q8K.pdb) (d) T5FEN (1UT5.pdb) (e) hEXO1 (3QEB.pdb) (f) DmXrn1 D207A (2Y35.pdb) (g) SpXrn2 (Rat1, 3FQD.pdb).

Figure 5. A unified mechanism for the FEN superfamily based upon integrated results plus conserved architectural and active site elements

(a) A model for FEN recognition of its substrate based on combined structural and biochemical results. The cap and gateway are disordered in the absence of DNA (model based on 1UL1.pdb). Initial DNA binding is through the K^+ /H2TH motif. The 5'-flap is threaded through the disordered gateway and cap. Proper binding of the substrate structural features facilitates ordering of gateway and cap. The nucleotides flanking the scissile phosphate then unpair to position the scissile phosphate for incision. Colouring is as in Figure 1. (b) Models for EXO1, XPG and GEN1 substrate recognition. The DNA 5'-nuclease superfamily members (cartoon) show common elements (extended C-termini omitted). For XPG, we predict the >700 residue R domain region (light orange) replaces the FEN1 cap to leave the gateway uncapped, allowing insertion of one ssDNA region of the DNA bubble. For GEN, the cap is absent, leaving the gateway uncapped for insertion of a HJ. A model where two GENs, rotationally related, are placed with the active sites facing each other is consistent with two GENs acting on one HJ and would minimize HJ opening.

(c) The XRN 5'-exoribonucleases conserve active site residues and substrate binding motifs of the FEN superfamily. Comparison of hFEN1-DNA (3Q8K.pdb), DmXRN1 D207A-DNA (2Y35.pdb) and SpXRN2 (3FQD.pdb, Rat1) structures. The overall fold of the XRN nuclease core domains and the structural elements are similar to hFEN1 (coloring as in Figure 1). Close up back views of the gateway and cap regions (partly disordered in the XRN) shows the similar positioning of architectural elements, conserved residues and DNA. Portions of the proteins behind the gateway and cap including the C-terminal region of the XRN were removed to allow this view.